miR-29b Mediates Anti-Cancer Effects of Psoriasin/S100A7 Overexpression in MCF-7 Breast Cancer Cells

Research Thesis

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by

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Abstract:

Breast cancer is the second leading cause of cancer mortality in women in which one in five women will develop the disease in their lifetime. Poor prognosis is associated with invasive and metastatic breast cancer. S100A7/Psoriasin gene is overexpressed in a wide range of epithelial malignancies, specifically ERα- breast cancer. MicroRNAs (miRNA) are short nucleotide sequences that modulate gene expression through partial complementary binding to 3' untranslated region (UTR) region of messenger RNA post-transcriptionally to either repress translation or degrade RNA. Dysregulation of miRNA expression is associated with cancer progression. miR-29b is a potent miRNA which has been shown to exert tumor suppressive properties. We have observed upregulation of miR-29b expression in S100A7-overexpressing MCF-7 breast cancer cells. The goal of my project is to understand how overexpressed miR-29b in S100A7-overexpressing MCF-7 cells affects proliferation and metastasis.

We created hS100A7 overexpressing MCF-7 cells by stably transfecting S100A7-containing vector. We observed significantly reduced cell proliferation in the overexpressing cells compared to vector control. Real-time PCR (qPCR) showed increased levels of miR-29b expression in S100A7-overexpressing cells compared with vector control cells. This is in accordance with our observation that NF-kB activation and nuclear translocation is inhibited by S100A7 overexpression, given that NF-kB is a negative regulator of miR-29b transcription.

Furthermore, Western blot analysis indicated S100A7 overexpression increased p53 protein level, which could be at least in part due to miR-29b upregulation. Thus, we verified the anti-proliferative effects of S100A7 upregulation in MCF-7 breast cancer cells is mediated through increased levels of miR-29b. Because malignant cells depend on dysregulated expression of miRNA genes, miR-29b can be further investigated as an effective miRNA-based therapy for cancer patients.

In conclusion, here we report that miR-29b is a potent miRNA that has been shown to exert tumor suppressive properties in ER+ breast cancer cells. S100A7-overexpressing cells presented anti-proliferative phenotype when compared with the vector via modulating key proteins important in cell-cycle regulation and cell metabolism. Our current model suggests that S100A7 overexpressing negatively regulates NF-κB which in turn results in increase in miR-29b levels and subsequently anti-cancer properties.

Introduction:

Breast cancer is the second leading cause of cancer mortality in women in which one in five women will be diagnosed with the disease.[1,2] Nearly one million breast cancer cases are diagnosed worldwide every year.[11]

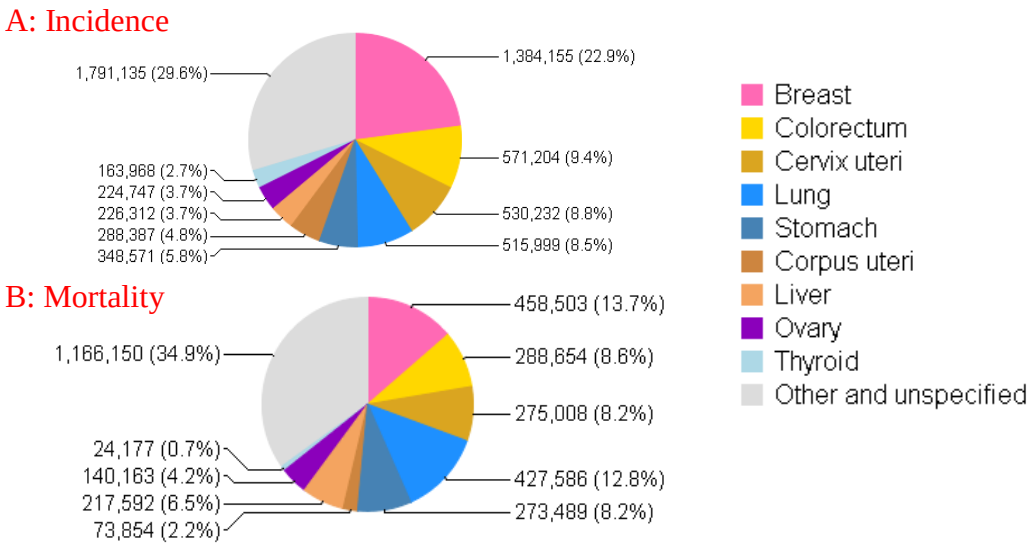

Figure 1. Breast Cancer Incidence and Mortality Statistics in Women Worldwide.[11] Breast cancer is the leading form of invasive cancer among women worldwide in both incidence and mortality rates. Non-invasive, non-melanomal skin cancers are included in these statistics under the 'Other and unspecified' categorization.

Despite advances in its early detection, about 30% of patients with early-stage breast cancer have recurrent disease. Metastasis to the brain, bones, lungs, lymph nodes, and liver, not the primary tumor within the breast, leads to death. Metastatic cells generally have dysfunctional growth regulation mechanisms, undergo cell adhesion alterations, and migrate to distant organs via the blood and lymphatic vessels, leading to secondary tumor formation that represents the most devastating feature of breast and other cancers. Therapies that target the signaling pathways of cell movement and growth may inhibit breast cancer metastasis.[3] signaling pathways in the breast tissue are strongly influenced not only by genetic factors but equally so by hormones.[21] Although the molecular mechanism is poorly understood, there is a correlation between lifetime exposure to endocrines (estrogen and progesterone) and the development of breast cancer.[22] Presence or absence of estrogen and progesterone receptors yield different results in progression of cancer. Therefore, breast cancers can be described as either endocrine receptor positive, human epidermal growth factor 2 (HER2) positive, triple negative, or triple positive.[23]

S-100 protein family has been implicated in tumorigenesis and progression. They are low molecular weight calcium binding proteins with high helix-loop-helix conformation associated with protein phosphorylation, cytoskeleton remodeling, cell growth and epidermal differentiation, and inflammatory response.[4] Moreover, they are induced in the epidermis and isolated from psoriatic keratinocytes.[25] S-100 proteins are utilized as markers for epidermal growth and differentiation in certain breast tissues.[5] S100 calcium-binding protein A7, psoriasin, or simply S100A7, is a low molecular weight protein which is coded by *S100A7* gene, maps to chromosome 1q21.2-q22, is involved in epithelial cell maturation and inflammation. Hu *et al* cohort clinical study showed markedly high mRNA levels of psoriasin in non-small cell lung cancer, specifically lung squamous carcinoma compared with adenocarcinoma, and was associated with poor prognosis.[24] In addition, Kesting et al reported that S100A7 promotes oral carcinogenesis and tumor progression.[25] However, S100A7 has been identified as having differential roles in regulating estrogen receptor negative/positive (ER-/+) breast cancer. It is highly associated with ER- breast cancer phenotype in which it modulates tumor growth via upregulation of inflammatory pathways.[6,9] Sneh *et al* reported that downregulating S100A7 in ER- breast cancer inhibits EGF-induced migration.[26] S100A7 is highly expressed in invasive breast carcinomas and high grade ductal carcinoma *in situ* (DCIS). Psoriasin seems to enhance tumor growth through prosurvival mechanisms such as NF-κB and AKT pathways.[24,27] In ER+, however, it had been shown that S100A7 regulates tumor growth through modulation of β-catenin/TCF4 pathway.[7,8] The mechanism behind this remains unknown. In ER+/PR+/HER2- MCF-7 cells S100A7 upregulation causes anti-cancer effects.[9,10] The current study seeks to investigate the role of S100A7 in ER+ breast cancer cells.

S100A7 regulates translocation of nuclear factor kappa B (NF-κB).[53] NF-κB is a primary transcription factor that responds to extracellular stress signals, inflammation, and anti-apoptotic stimuli.[29] It is mainly sequestered in the cytosol through binding to IκBα**;** when activated, by chemicals such as oxidizing agents or cytokines, it translocates to the nucleus and promotes cell survival. NF-κB inhibitor IκBα inactivates the nuclear factor through binding and hiding the nuclear localization signal (NLS), thus keeping the protein inactive in the cytoplasm. Mutations in *NFKBIA* gene that codes IκBα result in chronic activation of NF-κB and transcription alterations. Hay et al reported that IκBα mutations were found in Hodgkin's disease biopsy

samples.[28] This explains the constitutive expression of NF-κB in these samples and indicates that IκBα may have tumor suppressor properties.

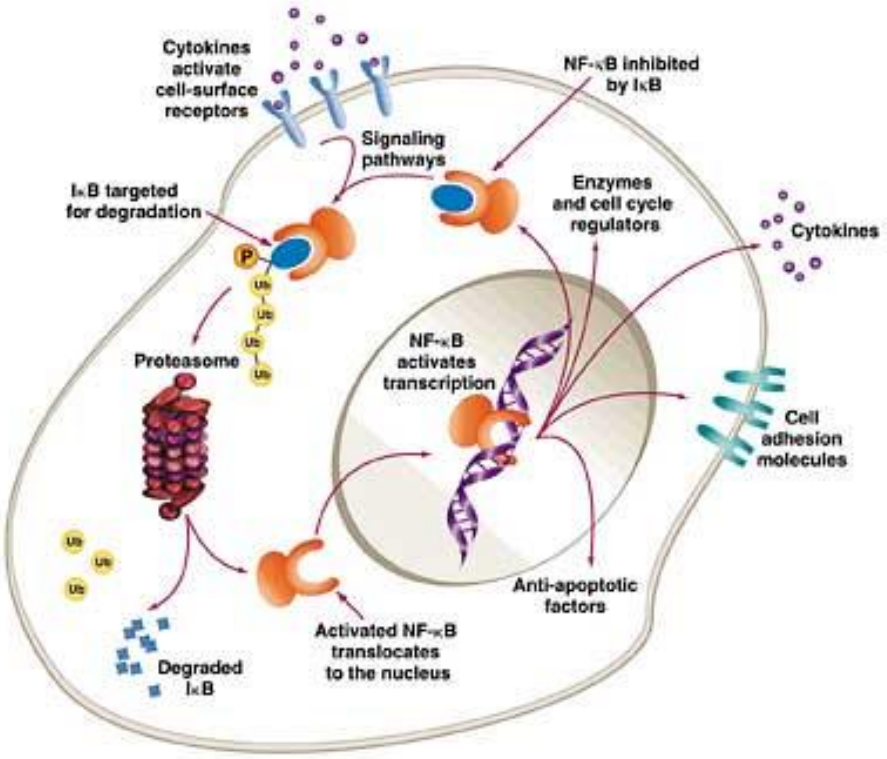

Figure 2. NF-κB Mechanism of Action.[30] The heterodimer NF-κB is located in the cytosol sequestered from cellular activities by inhibitory protein IκBα. Upon extracellular signaling and activation of integral receptors, the inhibitory protein is phosphorylated, subsequently ubiquitinated, dissociated from NF-κB and sent to the proteosome for degradation. Free, active NF-κB now binds nuclear receptors, translocates to the nucleus, and alters transcription through binding to response elements (ER) on the DNA promoting growth, inflammation, and cell survival.

Emberely et al reported a potential functional relationship between S100A7 and tumor progression through activation of intracellular signaling protein Jab1 (c-jun activation domain binding protein 1). Alterations in Jab1 protein expression and activation are associated with cell cycle dysregulation and tumor malignancies.[31] Through its existence as a single protein monomer or part of the complex constitutive photomorphogenic-9 signalosome (CSN5), Jab1/CSN5 complex is key in nuclear-to-cytoplasmic shuttling and transcription factor specificity. Jab/CSN5 signaling pathway is thought to be transcriptional coactivator and specificity factor for NF-κB, constitutive expression of B-cell CLL/lymphoma 3 protein which are involved in cell proliferation and apoptosis, respectively, and negative regulator of tumor suppressor p53 binding protein 1 (P53BP1) which is involved in DNA repair.[31-33] Onco-protein

Bcl-3, member of IκB inhibitory proteins, interestingly activates NF-κB transcription and promotes formation of NF-κB-DNA complex, thus signaling pro-inflammatory and cell survival pathways. S100A7 seems to be positively interacting with NF-κB in ER- breast cancer cells, thus acting as oncogenic gene, promoting tumor formation and associating with poor prognosis.[10]

Sneh et al and others demonstrated that S100A7 regulates epidermal growth factor (EGF) and EGF receptor (EGFR)-mediated signaling pathways. S100A7 downregulation corresponded to inhibition of EGFR-mediated signaling in ER- breast cancer cells.[26] Silencing S100A7 by S100A7 small hairpin (shRNA) inhibited EGF-induced cell migration and invasion. High EGFR levels are associated with metastatic and invasive forms of cancer. EGFR dimerization activates its intracellular tyrosin kinase activity, effectively initiating a plethora of signaling cascades inducing growth, proliferation, and migration. S100A7 activation of EGFR increases Class I phosphoinositide 3 kinase (PI3K) which in turn recruits protein kinase B (PKB, or Akt) promoting anabolic signaling pathways. Akt binds PI3K-activated, plasma-membrane-docked proteins, namely phosphatidylinositol-(3,4,5)-triphosphate (PIP3) and phosphatidylinositol-4,5-bisphosphate (PIP2). Akt is fully activated through double phosphorylation on its threonine 308 and serine 473 by phoshoinositide-dependent kinase-1 (PDK1) and the mammalian target of rapamycin Complex 2 (mTORC2), respectively. Effects of activated Akt on known targets are to inactivate them, ultimately to enhance cell survival. Watson et al reported that Jab1 nuclear expression levels were especially high in EGFR+ and S100A7+ tumors.[34]

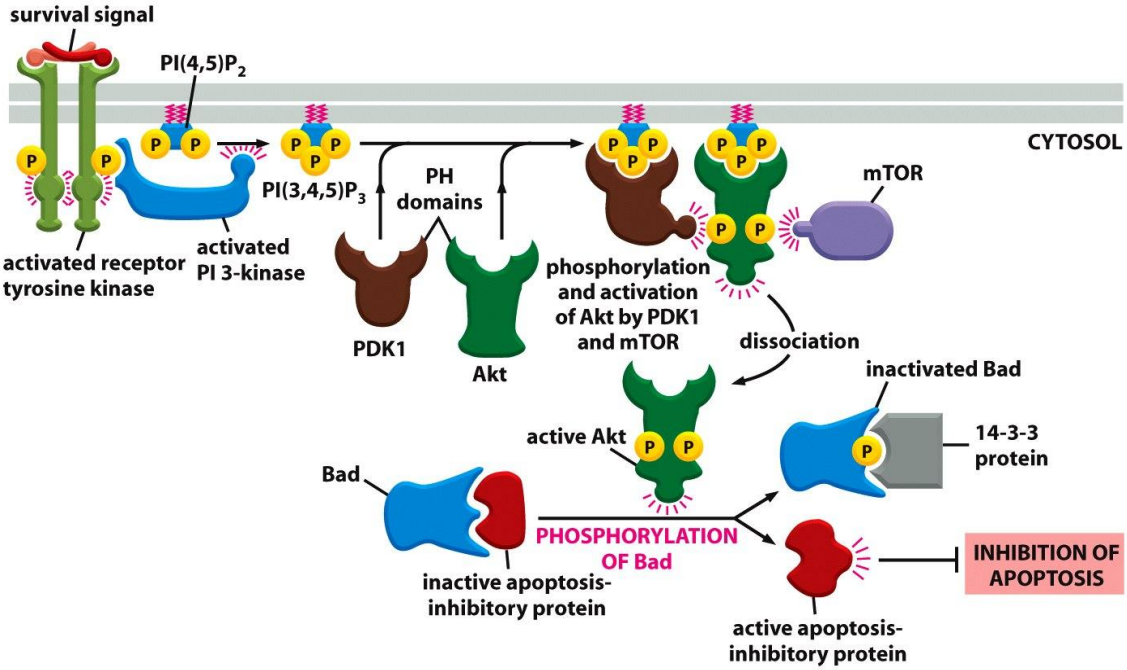

Figure 3. Akt-Mediated Cell Survival Through PI3 K Signaling. An extracellular survival signal – i.e. S100A7 in ER- breast cancer cells – activates a receptor tyrosine kinase (RTK), such as EGFR, which recruits and activates PI3 K. Activated PI3 K produces PIP3 which serves as a docking site for two serine/threonine kinases with pleckstrin homology domains – Akt and PDK1 – which are brought in close proximity to the plasma membrane. Akt is phosphorylated by mTOR, and subsequently double phosphorylated by PDK1 to be fully active. Activated Akt dissociate from the plasma membrane and goes on to phosphorylate other downstream target proteins important in cell survival and proliferation.[36]

While S100A7 positively regulates NF-κB in ER- breast cancer cells, it acts as tumor suppressor in ER+ breast cancer cells. NF-κB translocation is inhibited and consequently cells exhibit apoptosis, reduced migration and invasion. Our lab and others reported anti-proliferative, anti-migratory effects of S100A7 in ER+ breast cancer cells through the beta-catenin (β-catenin)/T-cell factor 4 (TCF4) protein pathway and enhanced interaction between β-catenin and E-cadherin – transmembrane protein mediates cell-cell adhesion and recognition. Hyperactivation of β-catenin/TCF4 pathways is a marker of most types of malignancies. This is due to stabilization and nuclear translocation of β-catenin where it binds transcription factors of TCF family converting them from transcription repressors to activators which ultimately activates a downstream cluster of oncogenic genes. Our lab has shown that overexpression of S100A7 in ER+ cells inhibited growth and metastasis *in vitro* and tumour growth *in vivo* through β-catenin/TCF4 pathway.[8] It was previously shown that S100A7 and β-catenin negatively regulate each other in squamous cell carcinoma of the oral cavity (SCCOOC).

β-catenin, encoded by *CTNNB1* gene, regulated cell to cell adhesion and gene transcription. Its aberrant and constitutive expression in the nucleus is associated with many types of cancer such as hepatocellular carcinomas, ovarian, breast, and colorectal cancer. Protein levels are regulated through ubiquitinylation and proteosomal degradation. When translocating to the nucleus, β-catenin binds TCF4 transcription factor activating regulator genes such as c-Myc and CyclinD1 inducing anti-apoptotic and proliferative effects. β-catenin is signaled for degradation upon double phosphorylation by the constitutively expressed Glycogen Synthase Kinase 3 alpha and beta (GSK3α/β) on the threonine-41 and serine-37 sites. In vitro, Ganju *et al* reported that S100A7-overexpressing cells shown reduced phosphorylated, inactive form of p-GSK3β and elevated levels of non-phosphorylated form which is indicative of its negative regulation of β-catenin. Akt levels, inhibitor of GSK3β, were decreased upon S100A7 overexpression. So, presence of estrogen receptor may not only modulate proto-oncogenes such as c-Myc and CyclinD1 but also regulate S100A7 effects on upstream signaling targets such as EGF-induced PI3K activation.

Short noncoding RNA molecules called microRNAs (miRNAs) seems to affect the expression of at least one third of all human protein-coding genes. The effects of S100A7 could be, at least in part, mediated through specific crosstalk of certain microRNAs. Dysregulation of miRNA expression is associated with cancer progression. Previous studies reported differential expression of different miRNAs in breast cancer subtypes which carried tumor suppressive and promoting characteristics.[42-44] It appears that the more than 400 different miRNAs present in human genome play a major role in post-translational regulation of different oncogenic and tumor suppressing proteins. This regulation is affected by tumor environment and other present proteins such as cell-adhesion components. When classic genome-wide studies of breast cancer mRNA levels compared with miRNA array studies, a correlation is found and generated a signature discriminating between benign and invasive breast cancer tissues.[46] Caldas et al demonstrated the modulatory role of miRNAs on mRNAs in breast tumors devoid of somatic copy-number aberrations (CNA-devoid).[45] Profiling studies of breast cancer cell lines reported that the vast majority of them presented deranged miRNA expression when compared with normal cells.[47]

MicroRNAs (miRNA) are short nucleotide sequences with a characteristic secondary structure of stem-loop, or hairpin, that modulate gene expression through antisense

complementarity to 3' untranslated region (UTR) of messenger, RNA (mRNA) post-transcriptionally to either repress translation or degrade RNA.[18] This class of non-coding RNAs was first identified in *Caenorhabditis elegans* as products of *lin-4* and *let-7* genes that are important in temporal postembryonic development. Mature miRNA are created from miRNA precursors which are first transcribed as a primary transcript (pri-miRNA). The pri-miRNAs are cleaved by the nuclear Class 2 RNAse III enzyme Drosha creating ~70 nucleotide long stem-loop molecules called pre-miRNAs.[12] Those pre-miRNAs are exported out of the nucleus to the cytoplasm via the shuttle protein exportin-5 to be processed by Dicer enzyme into mature, 18-24 long nucleotides, miRNAs.[19, 20] After being capped and polyadenylated, depending on their 5' end stability, miRNA strands bind protein argonaute-2 (AGO2). Along with other proteins, miRNA and AGO2 create what is known as RISC (RNA-induced silencing complex) that seeks out its mRNA targets through complementary nucleotide binding. In animals, seven nucleotide base-pairing is enough to initiate mRNA regulation usually on the 3' end. If binding is strong, then argonaute protein cleaves mRNA by removing its poly-A tail exposing it to exonucleases. If binding is less strong, then argonaute protein does not break the mRNA but rather shortens its poly-A tail which translates to destabilization of mRNA strand and ultimately translation repression. In this scenario, translation is repressed because mRNAs become cytosolic structures called processing bodies (p-bodies), sequestered from ribosomes, decapped, and finally degraded. P-bodies are dynamic structures, containing mRNA and RNA proteases, within the cytosol and the site in which cell is believed to degrade mRNAs. In the end, a single miRNA can regulate a set of mRNAs as long as they contain similar binding sequence. Therefore, miRNAs are very important regulators of gene expression and disease progression.

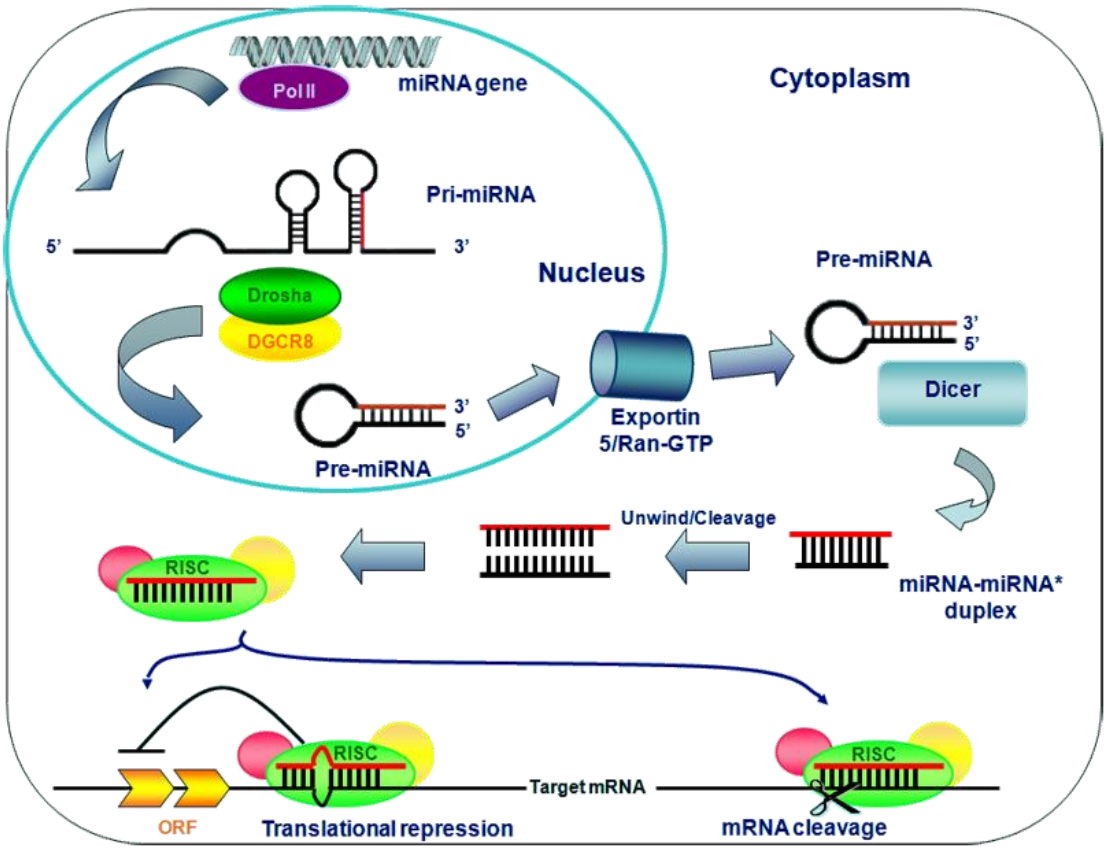

Figure 3. MiRNA Biogenesis: miRNA biogenesis begins inside the nucleus while its processing and maturation takes place in the cytoplasm of a eukaryotic cell. Long pri-miRNA transcripts, transcribed by RNA Polymerase II, are cleaved by the enzyme Drosha to produce smaller fragments (pre-miRNAs). These precursors are transported outside the nucleus via Exportin 5/Ran-GTP complex and are further processed by RNAse III Dicer which generates the double-stranded mature miRNAs. The strand corresponding to the mature miRNA is incorporated into a large protein called RISC (RNA-induced silencing complex) in which it will facilitate the binding to the 3' UTR of the target mRNA: perfect complementary binding induce mRNA cleavage, where partial binding represses translation.[20]

The miR-29 family is thought to exert regulatory roles in the cell through partial complementary binding to miRNA recognition elements (MREs) in the 3' UTR of target transcript. Laere *et al* reported in a study of 20 inflammatory breast cancers downregulated levels of miR-29 family in one of the most aggressive forms of locally advanced breast cancer.[48] In addition, Buffa *et al* demonstrated that expression of miR-29 family is associated with good prognosis.[49] Experiments have shown that some of miR-29 targets are the regulation of anti-apoptotic member of the B-cell lymphoma 2 family (Bcl-2), oncogenic DNA methyltransferases (DNA MTases), cell division control protein 42 homolog (Cdc42), phosphatidylinositide 3-kinase (PI3 Kinase), proto-oncogene tyrosine-protein Src (c-Src). [14,17,16, 52] Cdc42 is a small, conserved GTPase of the Rho family that is important in mammary gland morphogenesis, actin

polymerization, and cell cycle regulation. Bray *et al* reported elevated levels and aberrant expression of Cdc42 in breast cancer cells that are associated with increased motility, migration, and enzyme remodeling.[54] So, bringing Cdc42 levels down could lead to a better prognosis and thus it is an area of active research. Furthermore, mutations in the tumor suppressor gene p53 remain the most common in human neoplasia. Loss of p53 is associated with very poor prognosis, aggressive forms of breast cancer, and high migration rate. p53 is incredibly important in limiting neoplasm formation as observed in those expressing wild type p53. It is involved in, either directly or indirectly through downstream targets, DNA repair, regulation of apoptosis, inhibition of metastasis, and cell-cycle.[56] Restoring p53 in cancers is a major goal of active research. We report here that miR-29b is a potent miRNA which has been shown to exert tumor suppressive properties in ER+ breast cancer cells. It is significantly upregulated in S100A7-overexpressing MCF-7 breast cancer cells.[10] It is conceivable that it acts directly, or maybe indirectly, on the aforementioned targets to affect cancer development. Expression of miR-29b is negatively regulated by NF-κB nuclear translocation and c-Myc binding to its promoter region miR29-b-1/miR29-a.[55] Moreover, miR-29b expression can be suppressed through other signaling pathways such as hedgehog and inflammatory pathways. So, in our MCF-7 breast cancer cell model, it is conceivable that S100A7 inhibition of NF-κB could result in an increase of miR-29b transcription and less cancer phenotype.

Materials and Methods:

- **Cell Culture**

To analyze the effect of miR-29b on ER+ breast cancer cells, we used MCF-7 ER+ breast cancer cell line with high metastatic potential. Effects of miRNAs were analyzed in S100A7-overexpressing cells. All cells were obtained originally from ATCC, USA. MCF7-S100A7 overexpressing cells were cultured in complete medium (Dulbecco's modified Eagle's medium (DMEM), 10% heat inactivated fetal bovine serum (FBS)*, 1% penicillin-streptomycin). Cells were split every 36 – 72 h, depending on the growth rate of the cell line through centrifugation and dispersion into fresh media. The cells were kept in a humidified incubator containing an atmosphere of 5% CO_2 and 95% air at 37 ^{o}C. Cells were briefly rinsed with 0.25% trypsin-0.53 EDTA solution, then about 2 ml of the solution was added to the container and placed in the

incubator for 5 minutes. Trypsin-EDTA solution is neutralized by adding equal amount of complete DMEM. Cells are then collected, spun down, and added to fresh media in a new dish.

*FBS was heated to 60 ^{o}C for 30 min to inactivate proteins that might interfere with cell culture or any assays that FBS is used in.

- **Generation of S100A7-overexpressing Cells**

Open reading frame (ORF) of *Homo sapien*s-S100A7 homolog obtained from OriGene Technologies and subcloned into pIRES2-EGFP. MCF7 cells were transfected with pIRES2-EGFP plasmid clone or containing S100A7 with lipofectamine according to manufacturer's protocol (Invitrogen).[39] After 24 hours of transfection, cells were incubated for 3 weeks in medium containing G418 (500 μg/mL) to select the stably over-expressing S100A7 clones.

- **Gene Functional Enrichment**

Data analysis of the S100A7-overexpressing MCF7 breast cancer cells microarray data was conducted utilizing DAVID Bioinformatics Resources.[50]

- **RNA Extraction, Isolation, and Purification**

TRIzol reagent (Life Technologies) was used to extract total RNA. 1 mL was added per 100mm dish and incubated on ice for 3-4 m. Cells were scraped off, put in eppendorf tubes, and incubated at room temperature for 5 m. 0.2 mL chloroform was added (per 1 mL TRIzol) to samples; they were then mixed thoroughly for roughly 15 seconds until a cloudy solution is obtained. Samples were spun down at maximum speed at 4 degrees for 5 m. Upper aqueous phase was decanted into new tubes with 0.5 mL isopropanol (per 1 mL TRIzol) and incubated at room temperature for 15 m after mixing for 15 seconds. Samples were spun down maximum speed at 4 degrees for 10 m. Supernatant was removed and pellet was carefully washed twice with 75% ethanol; they were spun down at maximum speed at 4 degrees for 5 m. After pellets was air dried, they were rehydrated in 30-40 μL RNAse free water. RNA Purification of S100A7-overexpressing cells was performed using the RNAeasy Mini Kit (Qiagen).[41]

- **Real-Time Polymerase Chain Reaction (RT-PCR)**

PCR was used to genetically characterize effects of S100A7 on miR-29b expression in S100A7-overexpressing cells compared with normal cells via the use of gene-specific primers. Experiments followed Power SYBR Green Master Mix protocol of Applied Biosystems.[57]

- **siRNA Treatment**

miR-29b knockdown treatment was carried out in accordance with the manufacturers procedure.[51] Cells

- **Protein Isolation and Western Blotting**

S100A7-overexpressing cells were lysed with ice-cold radio immunoprecipitate assay (RIPA) lysis buffer* containing phosphatase and nuclease inhibitors. After 5-10 min, lysed cells were scraped off of the plate and transferred to Eppendorf tubes on ice which were then let to rotate for 30 min at 4 ^{o}C, centrifuged at 12,000 RPM at 4 ^{o}C, and the pellet was discarded. Remaining lysate was used for immediate protein estimation or stored at -20 ^{o}C for later protein estimation. Protein estimation was done according to modified version of the Microplate Assay Protocol (Bio-Rad)[37].

The volume of each sample to be prepared for resolution on the gel can be estimated by dividing the desired amount of protein (ng) by the value of the optical density. A final amount of 50 ng protein was prepared according to manufacturer's instructions (Invitrogen)[37]. 1X running buffer was prepared from NuPAGE® MES Buffer (10X) and add 250µL NuPAGE® Antioxidant for every 1L buffer. Denatured samples and 12µL Precision Plus Protein Dual Color Standard (BioRad) were loaded into a pre-cast 4-12 % Bis-Tris polyacrylamide gel and run at no more than 180V. The Bio-Rad Semi-dry Transfer Cell system was used to transfer separated proteins to a nitrocellulose membrane at 16V for 60 min for 1 blot or 70 min for 2 blots. The membrane was blocked using 5% non-fat dry milk in Tris-Buffered Saline Tween-20 (TBST) for 30 min. Primary antibodies were incubated overnight at 4 ^{o}C with shaking and secondary antibodies were incubated for 1 h at RT with shaking.

*RIPA buffer is prepared using the following recipe[38]: 150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), and 50 mM Tris, pH 8.0.

- **Viability and Proliferation Assay**

MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide is a clear yellow dye that, once added to viable cells, is metabolized by a mitochondrial enzyme, yielding a dark blue formazan compound. This compound is dissolved to a homogenous mixture by addition of the proprietary color development solution. The optical density (OD) can be measured at 570 nm and used to determine a ratio of viable to non-viable cells[40]. MCF7 cells were treated with 100, 200, and 300 nM siRNA, according to Mirus Bio *Trans*IT-TKO kit protocol, or vehicle and plated in 96-well flat-bottom plates.[51] Each concentration was tested in triplicate or quadruplets and enough wells were filled to test cytotoxicity over a five day period. To prepare reagents, 10 mL pH 7.4 PBS must me mixed with 1 vial MTT (50 mg MTT/vial). Cells were allowed to incubate in a 37 ^{o}C, humidified, 5 % CO_2 environment for 24 h before "day 1" cells were tested. MTT was added (0.01 mL) to the wells to be tested and allowed to incubate 4 h before adding 0.1 mL color development solution (isopropanol with 0.04 N HCl). Optical density can be read within an hour at a test wavelength of 570 nm and a reference wavelength of 630 nm. This procedure was repeated over five days.

Results:

- **Relative Expression of S100A7 in MCF7 Cells**

To study the effects of S100A7 expression on ER+ MCF-7 breast cancer cells, we generated a stable S100A7-overexpressing MCF-7 cell line **(Figure 3)**. MCF7 cells were transfected with pIRES2-EGFP plasmid clone containing S00A7 with lipfectamine according to protocol (Invitrogen)[39]. Stable clones were selected to carry out the experiments. Gene overexpression was measured using real-time PCR relative housekeeping gene, GAPDH, which is abundantly expressed in control and clones. There was 413 fold increase in S100A7-overexpressing cells compared with vector control.

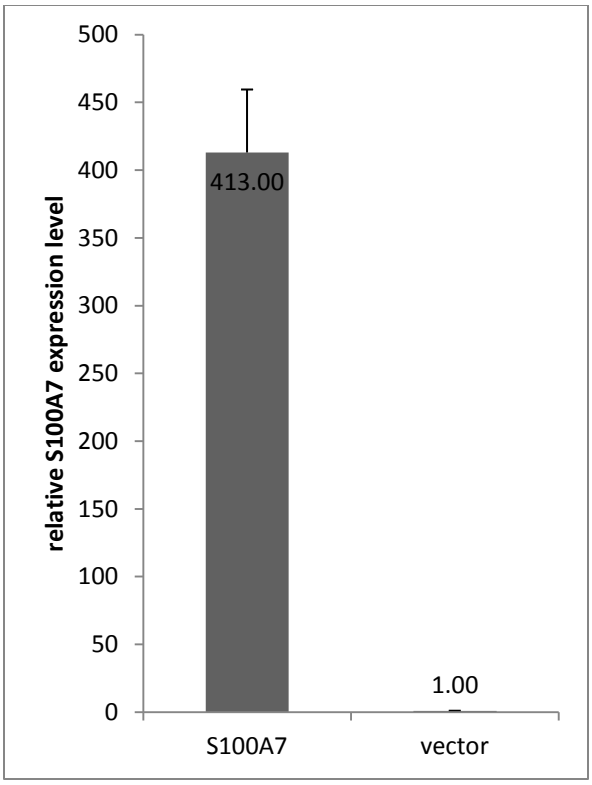

Figure 3. S100A7 Expression in MCF7 Cells: S100A7 gene expression relative to reference gene glyeraldehyde 3-phosphate dehydrogenase (GAPDH) in S100A7-overexpressing MCF7 cells compared with vector.

- **S100A7 Affects miR-29b Expression in MCF7 Cells**

In order to delineate the role of S100A7 overexpression, we ran a microarray experiment in which we found out that 167 genes were significantly downregulated more than 2.5 folds (p-value 0.0048) in S100A7-overexpression cells when compared with the vector **(Figure 4)**. We sought to understand the functional importance of these genes by running a Gene Functional Enrichment Analysis (GFEA) for S100A7-overexpressing MCF7 cells microarray data. GFEA analysis attributed 13% of those 167 genes were targets of miR-29b which indicates a correlation between S100A7 and miR-29b expression.

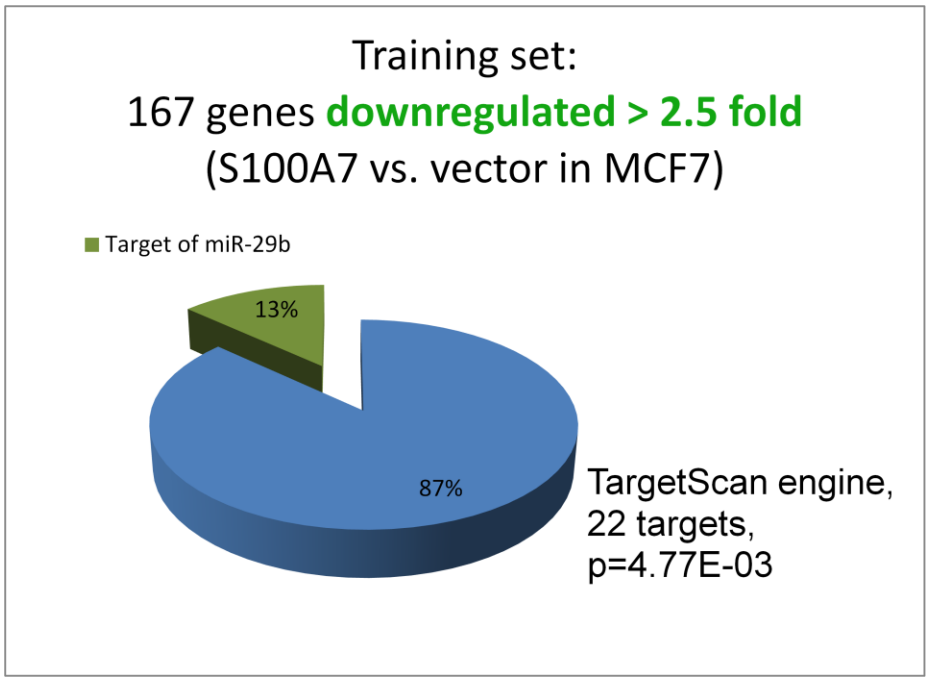

Figure 4. S100A7 Modulates Expression of 167 Genes in S100A7-overexpressing Cells Compared to Vector: Microarray data shows that S100A7 overexpression in MCF7 cells downregulates 167 genes more than 2.5 folds. Upon GFEA analysis, about 13% of these genes are targets of miR-29b (p-value 0.0048) which may indicate a relationship between S100A7 and miR-29b expression.

- **miR-29b Expression in S100A7-overexpressing MCF7 Cells**

After running GFEA analysis, we sought to further investigate what appeared to be a correlation between S100A7 and miR-29b expression. We ran a real-time PCR to analyze and quantify relative miR-29b expression in S100A7-overexpressing cells compared with the vector. We show here that primary transcript of miR-29b is relatively increased more than 7 folds in S100A7-overexpressing when compared with the vector **(Figure 5-A)**. In addition, our experiments show that the mature, functional miR-29b expression is about 11 folds more relative to RNU6B (housekeeping miRNA gene) in S100A7-overexpressing cells when compared with the vector **(Figure 5-B)**. Expression of pri-miRNA and mature miRNA was measured relative to endogenous reference gene RNU6B.

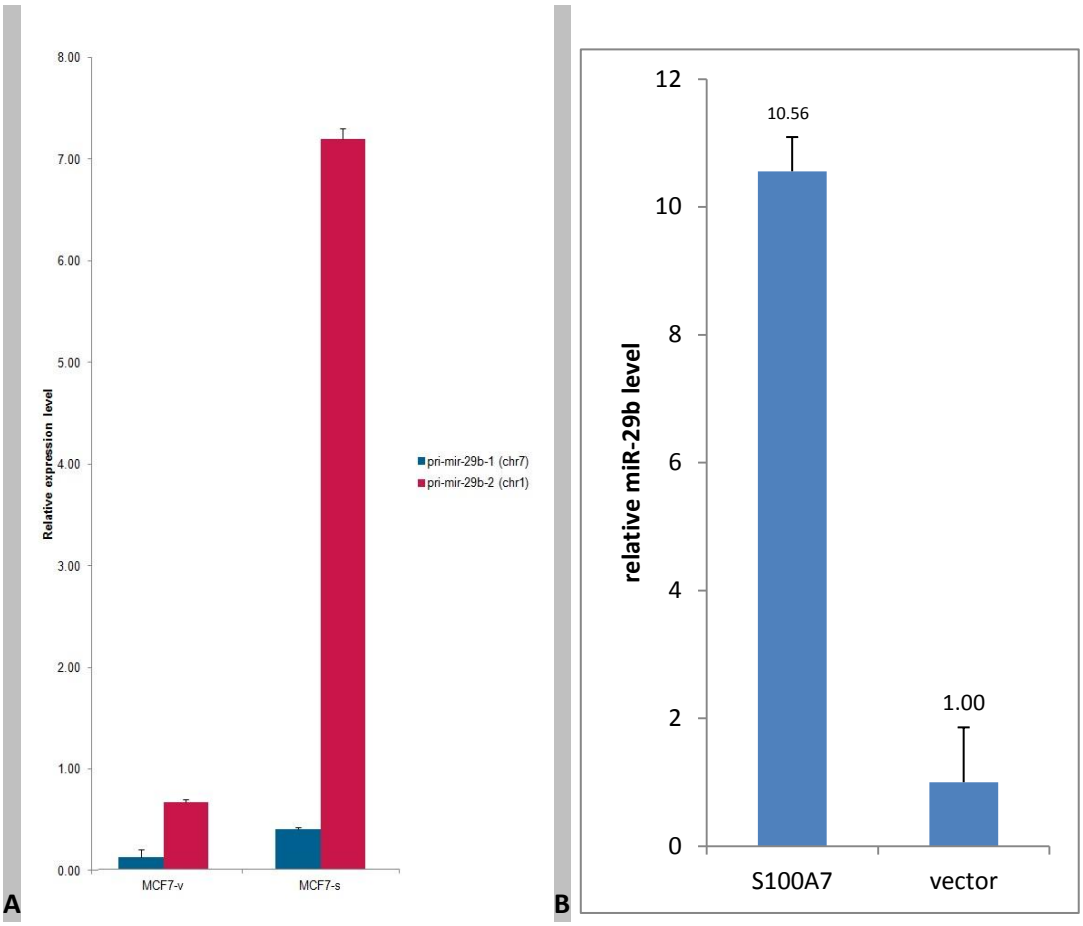

Figure 5. S100A7 Positively Regulates Expression of miR29b in S100A7-overexpressing MCF7 Cells Compared with Vector: miR-29b relative expression is affected in S100A7-overexpressing MCF7 cells compared with vector. S100A7-overexpressing cells showed marked increase of more than 7 folds in primary miR-29b transcript expression relative to the control (**A**). As for the mature, functional miR-29b, its expression is also increased by almost 11 folds relative to control (**B**)

- **S100A7 Effects on Downstream Tumorigenic Targets**

Now that we established a relationship between S100A7 and miR-29b expression we sought to identify any potential downstream targets that S100A7 would effectively modulate. We carried out Western blot experiments to identify such targets. We found that in S100A7-overexpressing cells p53, tumor suppressor, was increased relative to the vector control. Moreover, we noted a marked decrease in p85α, regulatory subunit of PI3 Kinase, Cdc42, protein involved in regulation of cell cycle, and GAPDH, which is important in cell metabolism. In addition, in order to understand whether miR-29b single handedly regulates these targets, we knocked down miR-29b in S100A7-overexpressing cells, carried out same Western blotting, and compared results with the control S100A7-overexpressing with miR-29b intact. Upon inhibiting miR-29b we saw

reverse trend in p85α and Cdc42 when compared to the control **(Figure 6)**. Levels of expression were assessed relative to the control β-actin which is abundantly expressed in both S100A7-overexpressing cells as well as the vector control. miR-29b was inhibited using complimentary oligonucleotides (300nM) that acted similarly to small-interfering RNA segments by forming a double stranded structure rendering miR-29b inaccessible to affect transcription.

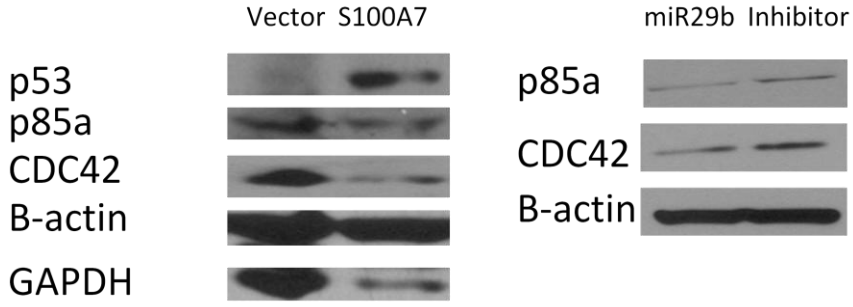

Figure 6. S100A7 Overexpression Affects Key Downstream Targets: S100A7 overexpression in MCF7 cells, relative to control, decreases all of which the regulatory subunit of PI3 K p85α, the metabolically important protein GAPDH, and the cell cycle division 42 protein (CDC42). S100A7 increases, however, tumor suppressor protein p53 in MCF7 cells relative to control. Reverse trend was observed in p85α and Cdc42 in the S100A7-overexpressing cells with miR-29b knockdown when compared with intact miR-29b S100A7-overexpressing cells. MiR-29b knockdown was done according to *Trans*IT-TKO protocol.[51]

- **miR-29b Knockdown Enhances Proliferation in S100A7-overexpressing MCF7 cells**

After establishing a connection between S100A7 overexpressing and miR-29b expression, we sought to investigate the proliferative affects of miR-29b on S100A7-overexpressing cells. In order to do that, we carried out viability assays in which we assessed whether or not miR-29b affected cell proliferation. Two groups of S100A7-overexpressing cells were analyzed; one was transfected with miR-29b complementary oligonucleotides to inhibit it according to Mirus Bio transfection kit protocol (300nM), and control with miR-29b intact.[51] After 48 hours of experiment, transfected cells were significantly higher in number than those with functional miR-29b (p-value 0.045). After 72 hours, difference in growth was not significantly different between the two groups.

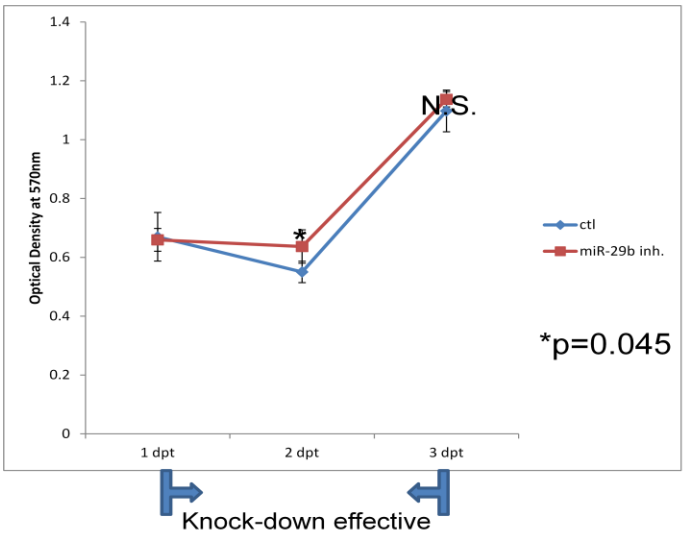

Figure 7. MTT Assay for miR-29b Knockdown in S100A7-Overexpressing MCF7 Breast Cancer Cells: S100A7-overexpressing cells were transfected with miR-29b inhibitor to assess cell viability and compare rates with the S100A7-overexpressing control that has functional miR-29b. The assay indicates that at 48 hr of treatment, at 300nM of miR29-b inhibitor, cells are growing significantly more when compared with vector (p-value 0.045). 25000 cells were added in triplets in 96-well tissue culture plates and the viability was measured after 0-3 days with MTT solution based on light absorbance.

Discussion:

Our lab had previously reported differential role of psoriasin/S100A7 gene in ER+/- breast cancer cell progression and metastasis.[10] In this study, we examined how S100A7 overexpression affects cell proliferation and tumor progression. Preliminary analysis of microarray data of S100A7-overexpressing cells when compared with control revealed 2.5 fold decrease in 167 genes. Genomic profiling indicated that 13% of these genes were targets of miR-29b family of microRNAs (p= 0.0048). We sought to further examine this relationship to understand the mechanism behind this. S100A7 overexpression corresponded to an increase in miR-29b gene expression. Previously, Sneh *et al* reported that S100A7 overexpression caused inhibition of NF-κB nuclear translocation, a pro-inflammatory transcription factor that promotes inflammation and cell survival pathways. So, although we have not tested this, but it is conceivable that the negative regulation of NF-κB, caused by S100A7, promotes miR-29b expression in ER+ breast cancer cells. We demonstrated that miR-29b expression modulates the expression of downstream effector proteins important in cell survival and tumor progression. We report here that S100A7 overexpression reduces the regulatory subunit of PI3 Kinase, p85α, an important indicator of cell survival pathways – akt-mediated cell proliferation specifically.[36] In

addition, S100A7 overexpression reduces cell cycle protein 42 (Cdc42) which is involved in cell regulation; also an important indicator of cell proliferation. More interestingly, we noted a decrease in GAPDH levels in S100A7-overexpressing cells when compared the vector control. It Since tumors exhibit high metabolic rates to meet their proliferative and metastatic needs, the observation is conceivable; however, the underlying mechanism is unknown. Furthermore, S100A7 seems to increase p53 protein levels which aligns with our current hypothesis in that psoriasin overepression reduces proliferation and decrease tumor growth. To understand whether or not these affects were miR-29b-mediated, we inhibited miR-29b and observed a reverse trend in two of the proteins, namely Cdc42 and p53. This concludes that miR-29b is directly related to expression of these two proteins. As for the other ones, it is unclear whether or not their expression was directly miR-29b mediated. We also report here that miR-29b mediated S100A7-overexpression reduced proliferation in miR-29b knocked down cells suggesting anti-proliferative effects.

Our current model suggests that miR-29b plays a tumor suppressor role in that it helps, at least in part, restoring p53 and Cdc42 which are important apoptosis and cell cycle regulators **(Figure 8)**. miR-29b also inhibits oncogene protein expressions thus conferring a reduction in tumor growth and proliferation which agrees with our experimental evidence as well as previous research. Understanding miRNA dysregulation aids in combating cancer and developing novel therapeutics. This study can be used as a cornerstone for further miR-29b studies that seek to develop drugs to kill cancer.

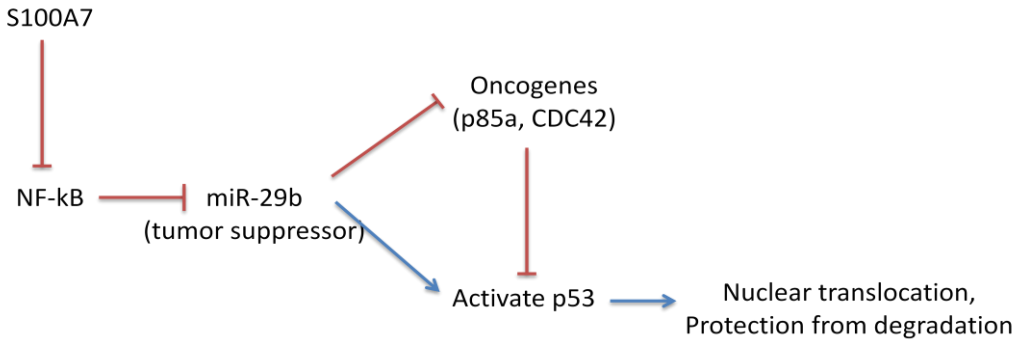

Figure 8. Proposed Hypothesis: Since S100A7 negatively regulates NF-κB, as previously reported, miR-29b is positively regulated by S100A7. miR-29b promote p53 and decrease, at least in part, p85α and Cdc42 protein expression thus rendering itself as a tumor suppressor. Therefore, S100A7-overexpressing cells show anti-cancer phenotype as supported by our experiments.

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